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EH3 (ABHD9) - the first member of a new epoxide hydrolase family with high selectivity for fatty acid epoxides

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Running title: New human epoxide hydrolase for signaling molecules

Abbreviations: ABHD, alpha-beta hydrolase; ACU, N-adamantyl-N'-cyclohexyl urea; AEPU, 1-((3S,5S,7S)-adamantan-1-yl)-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea; AUDA, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; AUOA, 8-(3-((3S,5S,7S)-adamantan-1-yl)ureido)octanoic acid; c-AUCB, 4-(((1S,4S)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid; CDU, 1-cyclohexyl-3-dodecylurea; EETs, epoxyeicosatrienoic acids; EH, epoxide hydrolase; peg1/MEST, paternally expressed gene 1/mesoderm specific transcript; t-AUCB, 4-(((1R,4R)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid; TPAU, 1-(1-acetylpiperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea; t-TAUCB, 4-(((1R,4R)-4-(3-(4-(trifluoromethoxy)phenyl)ureido)cyclohexyl)oxy)benzoic acid.

Abstract

Epoxide hydrolases are a small superfamily of enzymes important for the detoxification of chemically reactive xenobiotic epoxides and for the processing of endogenous epoxides that act as signaling molecules. Here, we report the identification of two human epoxide hydrolases, EH3 and EH4. They share 45% sequence identity, thus representing a new family of mammalian epoxide hydrolases. Quantitative RT-PCR from mouse tissue indicates strongest EH3 expression in lung, skin, and upper gastrointestinal tract. The recombinant enzyme shows a high turnover number with 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acid (EET), as well as 9,10-epoxyoctadec-11-enoic acid (leukotoxin). It is inhibited by a sub-class of N,N'-disubstituted urea derivatives, including 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, 1-cyclohexyl-3-dodecylurea, and 1-(1-acetylpiperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea, compounds so far believed to be selective inhibitors of mammalian soluble epoxide hydrolase (sEH). Its sensitivity to this subset of sEH inhibitors may have implications on the pharmacologic profile of these compounds. This is particularly relevant because sEH is a potential drug target and clinical trials are under way exploring the value of sEH inhibitors in the treatment of hypertension and diabetes type II.

Keywords: epoxyeicosatrienoic acid, blood pressure, pain, diabetes type II

Introduction

Epoxide hydrolases (EH) are enzymes that hydrolyze oxirane (epoxide) derivatives to the corresponding diols. They serve a plethora of vital functions, including detoxication of chemically reactive epoxides (1), formation of defense barriers by plants against herbivores (2), as well as the regulation of a large variety of physiological functions (3). The latter capability is due to the fact that a growing number of lipid-derived epoxides has been identified as signaling molecules in higher organisms. This is best documented for the arachidonic acid-derived epoxides, in particular the epoxyeicosatrienoic acid (EET) regioisomers, that have been shown to be involved in the regulation of e.g. blood pressure (4,5), pain perception (6,7), angiogenesis (8) and inflammation (9). Thus, pharmacologic intervention in the pathways controlling their biologic activity is an appealing target for drug development (10,11).

The vast majority of EHs belong to the structural family of α/β hydrolase fold enzymes and share the same three-dimensional structure and enzymatic mechanism (12), with few exceptions (13-15). Two mammalian α/β hydrolase fold EHs have been described and studied in detail. The microsomal epoxide hydrolase (mEH) is the major player in the defense against reactive, xenobiotic-derived epoxides (16). Recent results suggest that it also plays a role in signaling molecule processing (17). The sister enzyme soluble epoxide hydrolase (sEH) (18) serves a prominent function in the processing of important signaling molecules (19,20), in particular EETs. In addition, it complements the detoxification function of mEH by inactivation of reactive *trans*-disubstituted epoxides (21), that escape the otherwise very broad mEH substrate spectrum.

Like all α/β hydrolase fold enzymes, EHs hydrolyze their substrate via intermediate formation of an enzyme-substrate ester (22,23). Two structural features in the active site allow to discriminate EHs from the large pool of other α/β hydrolase fold enzymes: i) EHs possess an aspartic acid residue as the catalytic nucleophile to form said ester intermediate, and ii) they use two tyrosines in their lid domain to recognize and activate the substrate (for details on the enzymatic mechanism see Supporting information S1).

Based on such structural characteristics, we have identified three potential new mammalian EHs, and describe here the biochemical properties of one of these, namely EH3, in detail. This novel human EH, together with its relative EH4, forms a new family of mammalian EHs and displays a high turnover number with fatty acid-derived epoxides, indicating a role in the regulation of important physiological processes.

Results

Sequence similarity search reveals three new human candidate epoxide hydrolases

Previous sequence comparisons of epoxide hydrolase-related α/β hydrolase fold enzymes (22,24) revealed a highly conserved 16 amino acid sequence motif RVIAPDLRGYGDSDKP that was used as the bait in database searches for new epoxide hydrolase candidates. This resulted in the identification of 3 new human proteins, designated ABHD7, ABHD9 and peg1/MEST. Their amino acid sequences are highly conserved among mammalian species. Further inspection of these sequences proved the presence of all signatures necessary for and compatible with an epoxide hydrolase function (detailed in Supporting Information SI2). Sequence alignment of these proteins with other well characterized EHs revealed that ABHD7 and ABHD9 are most similar to a recently described set of soluble epoxide hydrolases from *Caenorhabditis elegans* (25) and represent a new family of mammalian epoxide hydrolases, due to a shared sequence identity of 45%. A phylogenetic tree indicating this relationship is shown in Fig. 1. Based on the results reported in the following we have proposed to rename the ABHD9 and ABHD7 proteins to EH3 and EH4 and the corresponding genes to EPHX3 and EPHX4, respectively, which was approved by the human genome nomenclature committee, and we use these designations throughout (for the rational behind this nomenclature see Supporting Information SI3).

EH3 is a functional epoxide hydrolase

We concentrated on the physicochemical and functional characterization of EH3 (ABHD9). The human gene has the chromosomal localization 19p13.12 and codes for a 1.8 kb mRNA composed of 7 exons. The respective mRNA codes for a 360 aa protein with a predicted molecular weight and isoelectric point of 40'909 Da and pI 7.7, respectively. Residues 22 to 44 are exclusively hydrophobic in nature and are predicted to represent a membrane insertion signal. They are followed by a stretch of arginine residues that potentially acts as a stop transfer signal, suggesting a cytoplasmic orientation of the protein, similar to that reported for mEH (26).

In order to investigate whether EH3 has the predicted catalytic function we recombinantly expressed the protein in *E. coli*. We failed to obtain detectable amounts when trying to express the complete protein. Instead, an N-terminally truncated version of EH3 carrying an N-terminal His-tag was found in appreciable amounts. The recombinant protein was obtained as inclusion bodies, and was therefore not useful for the analysis of enzymatic properties. We used it for rabbit immunization after purification and obtained an antiserum that was suitable for the detection of the protein by immunoblotting. Likewise, an attempt to obtain an enzymatically active EH3 via recombinant expression in yeast failed.

The recombinant full length enzyme was eventually obtained by baculovirus-mediated expression in insect cells. The yield of EH3 protein was low, routinely amounting to around 0.1 % of the total cellular protein, as proven by immunoblot analysis. In line with our prediction, the recombinant protein was

membrane-bound as demonstrated by the enrichment in the 100,000 x g pellet of the insect cell homogenate after differential centrifugation (Fig. 2).

The insect cell homogenate was used to assess the catalytic capabilities of the recombinant enzyme. Neither styrene 7,8-oxide, a generic substrate that is hydrolyzed by most EHs (27), nor cholesterol 5,6-epoxide, a substrate for a membrane-bound human EH of which the identification has been reported during the preparation of the present manuscript (28), was converted to the corresponding diol by EH3. In contrast, we did detect a high turnover of 9,10-epoxystearic acid with the recombinant cell lysate, an enzymatic activity that was essentially absent from mock-infected insect cell lysates.

Being now capable of assessing the functional integrity of the enzyme, we tried to solubilize it from the membrane in order to attempt its purification. However, multiple trials with different types of detergents and a variety of different experimental conditions did not lead to the release of detectable amount of catalytic activity into the 100,000 x g supernatant. Therefore, we are, at present, not able to obtain the enzyme in a purified, catalytically active form.

D173, Y220, Y281, D307 and H337 are the residues involved in EH3-mediated catalysis

Sequence comparisons (see Supplementary Information, Fig. SI2) suggested D173 as the catalytic nucleophile and H337-D307 as the charge relay system of the EH3 catalytic triad. Y281 and Y220 were the predicted candidates for the catalytic tyrosines in the lid domain of the enzyme. Y280 was, in addition, considered as a possible alternative because of its proximity to the

neighbouring Y281. In line with this, the respective mutants D173A, Y220F, Y281F, D307A, D307N, H337Q and H337A expressed in insect cells lacked any detectable hydrolytic activity with 9,10-epoxystearic acid (Fig. 3A), substantiating the importance of the five modified residues in substrate turnover (Fig. 3B). In contrast, the mutant Y280F displayed significant enzymatic activity, supporting that Y281 is the catalytic tyrosine and showing the lack of substantial effect of a single amino acid exchange when the respective residue is not directly involved in catalysis. In apparent contradiction to our working hypothesis, the mutant D173N showed a substrate turnover similar to that of the Y280F mutant. However, this is well compatible with the expected role of D173 as the catalytic nucleophile because self-activation by autocatalytic hydrolysis from the mutant asparagine to the wild type aspartic acid side chain has been reported for the equivalent mutants of other EHs (29,30).

EH3 is highest expressed in mouse skin, lung and upper gastrointestinal tract

To assess the expression pattern of the EH3, we isolated mRNA from a representative set of mouse organs and analyzed these by quantitative RT-PCR (Fig. 4). The strongest signals for EH3 expression were obtained with RNA from skin, lung, tongue, esophagus and stomach. Intermediate expression was found in pancreas and eye, followed by visceral fat, lymph nodes, spleen, aortic arch (used for normalization) and heart. Low signals were obtained with RNA from kidney, testis, ovary intestine, brain and liver. The lowest, yet still detectable, expression was found in skeletal muscle.

Epoxyeicosatrienoic acids and leukotoxin are endogenous substrates of EH3

The turnover of 9,10-epoxystearic acid raised the question whether other fatty acid-derived compounds can be hydrolyzed by EH3. We therefore looked at the turnover of EETs and leukotoxin as physiologically relevant substrate candidates. Indeed, EH3 efficiently hydrolyzed all these compounds. Fig. 5 shows the respective kinetic analysis. The derived kinetic constants are given in Tab. 1, in comparison with data obtained with recombinant purified human sEH and mEH under the same conditions. All analyzed substrates were turned over by EH3 with high V_{\max} , yet also comparatively high K_m , resulting in catalytic efficacies that are in the range of those obtained with sEH and mEH. The highest catalytic efficacy was observed with leukotoxin. Turnover of 5,6-EET could not be quantified because the insect cell preparation displayed a significant background activity with this substrate.

EH3 is inhibited by urea derivatives regarded as specific for sEH

In view of the fact that EET turnover by EH is evolving as a promising target for therapeutic intervention (11), and in particular inhibitors of sEH are presently developed as potentially marketable drugs (10), we were interested in a possible interference of such drugs with the EH3-mediated turnover of signalling molecules. We tested a subset of representative EH inhibitors for their inhibitory potency on EH3 catalysis at relevant concentrations (Tab. 2). The potent inhibitors specific for mEH (elaidamide) and sEH (ACU) were essentially not affecting EH3 activity. Likewise, a new generation of sEH inhibitors with bulky structure, represented by 4-(((1*R*,4*R*)-4-(3-(4-

(trifluoromethoxy)phenyl)ureido)cyclohexyl)oxy)benzoic acid (t-TAUCB), 4-(((1S,4S)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid (c-AUCB) and 4-(((1R,4R)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid (t-AUCB), did not show any effect. However, a group of inhibitors hitherto believed to be specifically targeting sEH activity reduced EH3 hydrolysis of 8,9-EET significantly at a concentration of 1 μ M. The most potent of these were 1-(1-acetylpiperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea (TPAU), 1-cyclohexyl-3-dodecylurea (CDU) and 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), popular sEH inhibitors widely used for *in vivo* animal studies. We studied their inhibitory profile for EH3 in more detail and determined their IC₅₀ (that is expected to equal K_i under the employed experimental conditions) to around 100 nM (Fig. 6), thus well in the range to have the enzyme affected under the experimental conditions usually employed for *in vivo* sEH inhibition (31).

Discussion

EH3 is a hitherto undiscovered mammalian epoxide hydrolase displaying a high turnover with fatty acid-derived epoxides, in particular EETs and leukotoxin, while being essentially inactive towards the generic EH substrate styrene oxide. This substrate selectivity suggests that the enzyme is implicated in processing of signaling molecules rather than detoxification of xenobiotic epoxides. It has the highest specific activity with EETs so far reported for an EH, and a catalytic efficacy being in the range of that of sEH, the major enzyme in EET turnover (20). Its expression pattern in the mammalian organism is quite different from that of the other well characterized EHs in that it is only poorly expressed in the liver but well expressed in skin, lung and upper gastrointestinal tract. Based on this observation, it is tempting to speculate on a potential role of EH3 in barrier formation, because the above tissues represent the major contact surfaces with the outside. Indeed, EH3 has been identified as a candidate disease gene for ichthyosis, based on comparative studies in human and mouse (32), which is compatible with such a function.

Furthermore, we find EH3 to be the most efficient catalyst for the hydrolysis of leukotoxin. The resulting metabolite has been reported to be a strong mediator of acute lung failure (ARDS; (19)) which suggests that EH3 might contribute to leukotoxin toxicity, in particular since we find a particularly high EH3 expression in lung (see Fig. 4).

Intriguingly, EH3 is sensitive to inhibition by N,N'-disubstituted urea derivatives, a class of compounds that has been developed as specific inhibitors for the mammalian sEH (33). In contrast to the latter enzyme, EH3

seems to be inhibited only by a subset of sEH inhibitors. Urea derivatives have been developed as EH inhibitors with the aim to pharmacologically interfere with the EET metabolism and have been proposed as potentially useful agents to treat high blood pressure (10), pain (7), and recently diabetes type II (34). Our present observation divides the urea derivatives into sEH selective inhibitors and mixed sEH and EH3 inhibitors. This has to be taken into account for the interpretation of *in vivo* data obtained with the different sets of compounds. Whether sEH-selective or the mixed-type blockade of EET hydrolysis is more favorable in the treatment of diseases remains to be established.

The expression of the EPHX3 gene has been reported to be down regulated in a variety of tumors. Hypermethylation in the promoter region, associated with a reduced transcription rate, was observed in gastric cancer (35), prostate cancer (36) and melanoma (37). In view of the recently described potential of EETs to reactivate dormant tumors and foster metastasis (38), possibly related to the angiogenic properties of EETs (39), this suggests a potential role of EH3 in the prevention of malignancies.

Finally, it is reasonable to speculate about why there are (at least) 3 separate EHs for the breakdown of bioactive fatty acid epoxides, namely EH3, sEH and mEH? Apart from the simple answer that these differ somewhat in their substrate selectivity, there is an additional intriguing hypothesis to come up with: if we accept that the hydrolysis products, like the DHET and the leukotoxin diol, have their own biologic activity (40), sometimes distinct and possibly opposite to the effects elicited by the parent molecules, the local ratios in the concentration of epoxide to diol may dictate the signaling

outcome. Among the several factors that influence this ratio, the above enzymes particularly differ in two of them, subcellular localization and K_m . The effect of the subcellular localization is obvious. The ER-resident EHs may have a significant kinetic advantage if they are expressed in the same cell as the epoxide-forming monooxygenase because they might directly hydrolyze the epoxide during their formation, yielding a high diol to epoxide ratio at low epoxide formation rates, as already discussed earlier for mEH (17). The major difference between mEH and EH3, the two ER resident EHs, is the enormous difference in their K_m for fatty acid epoxides, while their catalytic efficacies are within the same range (see Tab. 1). The resulting consequences are best explained by looking at a kinetic diagram of the enzymes with a common substrate, 11,12-EET (Fig. 7). While both enzymes should afford a similar diol to epoxide ratio at low epoxide formation rates, this ratio would strongly decrease once the substrate concentration increases over the low mEH K_m , while the ratio would stay constant with EH3, due to its extremely high K_m that lies even beyond the highest locally expected substrate concentration. Thus, EH3 would be the most suitable EH under conditions where a high diol formation rate with only a small EET leakage is desired.

Materials and Methods

Database screening for candidate epoxide hydrolases

Screening of the “build protein” database on the NCBI Human Genome BLAST website was performed using the BLASTP algorithm with the amino acid sequence RVIAPDLRGYGDSDKP as the search motif. Obtained hits were inspected for the presence of other sequence motifs indicative of an EH function (detailed in Supporting Information SI2).

Cloning and expression of EH3

Human EH3 cDNA was amplified from the I.M.A.G.E. consortium clone 2226429 (gene bank accession number AI570023). The full length cDNA was inserted into the pGEF II bacterial expression vector (29). A 5'-truncated cDNA lacking the coding region for the N-terminal 48 amino acid residues was cloned into the bacterial expression vector pRSET B (Invitrogen, Basel, CH) to obtain an anchorless, N-terminally His-tagged fusion protein. The resulting constructs were verified by sequencing and transformed into *E. coli* BL21AI for recombinant expression as described (41). For the expression in insect cells, the full length cDNA was inserted into the pFastBac plasmid (Invitrogen, Basel). Recombination with the baculovirus genome was achieved by transformation of the resulting pFastBac EH3 into *E. coli* DH10Bac. The resulting bacmid was purified, verified by PCR and sequencing, and used to transfect Sf9 insect cells to generate the intact recombinant baculovirus. Recombinant protein expression was accomplished by insect cell infection in suspension culture at a multiplicity of infection of 5. Five days post infection, cells were harvested. Lysates were obtained by a single pass through a

FrenchPress pressure cell (American Instrument Exchange, Haverhill, MA) at 30'000 psi and stored at –80 °C until use.

EH3 mutants were produced by mutating pFastBac EH3 via the Quikchange™ mutagenesis procedure (Stratagene, La Jolla, CA) and further processing as described above (for details see Supporting Information SI4).

Subcellular fractionation and immunoblot analysis

EH3 was purified under denaturing conditions by preparative coomassie blue-SDS gel electrophoresis (42) from inclusion bodies obtained with the pRSET construct and used to raise antisera in rabbits as described previously (43). The resulting serum has a detection limit of 0.5 ng of recombinant human EH3 per lane by Western blot analysis (44) at a dilution of 1:1000 using colorimetric detection (see below). To assess the subcellular distribution of EH3, insect cell lysates were subjected to differential centrifugation (10'000 x g for 20 min to pellet larger organelles, followed by 100'000 x g for 1 h to pellet membrane vesicles). Resulting fractions were analyzed by immunoblotting, using the EH3-specific rabbit antiserum (1:1000) and an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:10'000; Sigma, St. Louis, MO), followed by colorimetric detection using NBT/X-Phosphate. As a positive control for the distribution of ER membrane vesicles in the above procedure, insect cells infected with a recombinant mEH-coding baculovirus were used.

Enzyme assays

Enzymatic hydrolysis of 9,10-epoxystearic acid was assayed by a TLC-based procedure essentially as previously described (45), using a CycloneTM Storage Phosphor Scanner (PerkinElmer, Waltham, MA) for quantification of the radiometric signals. Hydrolysis of the different EET regioisomers was quantified in insect cell lysates by LC-MS/MS as described recently (17). Leukotoxin turnover was assayed under the same experimental conditions, using the mass transitions 295.2/171.1 and 313.2/201.1 for the quantification of leukotoxin and leukotoxin diol, respectively. Immunoquantification of EH3 in insect cell lysates is detailed in Supporting Information (SI5). For inhibition studies, EH3 lysates or purified human sEH were preincubated for 5min on ice with EH inhibitors at the indicated concentrations, prior to addition of the substrate.

Expression analysis of EH3 in mouse tissues

Tissues for mRNA analyses were taken from 12 week old C57BL/6 mice. Animals were sacrificed and organs were instantly removed by surgery and snap frozen in liquid nitrogen until further processing. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with the High Capacity cDNA Archive Kit (Applied Biosystems). Primer/probe sets for mouse Ephx3 (Mm01345663_m1) and GAPDH (Mm99999915_m1) were purchased from Applied Biosystems. Real time RT-PCR was run with Maxima qPCR Master Mix (Thermo Scientific), analyzed using the ABI Prism 7700 thermocycler (Applied Biosystems), and differential expression was calculated using the $\Delta\Delta$ CT method. Primer/probe based

expression values were validated by Sybr Green real time RT-PCR (Mouse EPHX3 Primers: 5'-tcccatgtcagtgatccaag-3' and 5'-tggaagtcagacatagacaacagc-3').

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Figure legends

Fig. 1. Phylogenetic tree of epoxide hydrolases. Boxes represent the linear amino acid sequence of human EHs (inside the gray rectangle) compared to their *C. elegans* counterparts. The location of the α/β hydrolase fold domain (gray shaded boxes) and the lid domain (black box) is indicated. Dark grey boxes represent (potential) N-terminal membrane anchor sequences. Sequence alignment was performed using ClustalW (46). The resulting dendrogram on the left side indicates the phylogenetic relation. Numbers at the bifurcations indicate sequence identity (mean sequence identity \pm standard deviation, in the case of multiple comparisons).

Fig. 2. Subcellular localization of EH3. Lysates of Sf9 cells expressing human EH3 or rat mEH, used as a microsomal marker, were subjected to differential centrifugation and subsequent immunoblot analysis. Immunoreactivity for EH3 and mEH is restricted to the 100,000 x g pellet, indicating their membrane association. Abbreviations used are : CL = control insect cell lysate; RL = recombinant insect cell lysate; P10 = 10'000 x g pellet; P100 = 100'000 x g pellet; S100 = 100'000 x g supernatant.

Fig. 3. A. Enzymatic activity analysis of EH3 mutants. Hydrolysis of 9,10-epoxystearic acid by the different mutants was assayed using thin layer chromatography for the separation of substrate (epoxide) and product (diol) of the enzymatic reaction. Identity of the respective mutant is given on top of each lane. Of all mutants analyzed, only Y280F and D173N display enzymatic activity. B. EH3 active site architecture, deduced from the structural

alignments and mutant analyses (for details on the enzymatic mechanism see Supporting Information 1).

Fig. 4. Expression pattern of EH3 gene in mouse tissues. mRNA isolated from different mouse organs was analyzed by reverse transcriptase-PCR using EH3-specific primers. Amplification of GAPDH mRNA served as the control. Expression levels in the individual tissues were normalized to those in the aortic arch. The bars represent the average obtained from four individual isolates, error bars indicate the standard deviation. The insert shows a magnification of the results obtained from tissues with lower expression yields.

Fig. 5. Kinetic analysis of EH3-catalyzed leukotoxin and EET turnover. Sf9 cell lysates expressing recombinant EH3 were analyzed for the hydrolysis of leukotoxin, 8,-EET, 11,12-EET and 14,15-EET, using substrate concentrations up to 50 μ M, dependent on the compound. Larger concentrations displayed significant deviations from the Michaelis-Menten kinetics, presumably due to micelle formation of the substrate. Modelling of the Michaelis-Menten kinetic (solid line) was performed using Prism 5 (GraphPad Software, San Diego, CA).

Fig. 6. Determination of IC_{50} ($\approx K_i$) of AUDA, CDU and TPAU. EH3 lysate was incubated with either of the three strongest inhibitors identified by the preliminary screening (see Tab. 2) for 5 min on ice prior to addition of 8,9-EET (5 μ M) and analysis of turnover. The enzymatic activity is expressed in %

of activity of the accompanying solvent control (no inhibitor). Kinetics were modelled using Prism 5. Note that under the present conditions (substrate concentration 7-fold below K_m), the IC_{50} essentially equals K_i ($K_i > 90\%$ of IC_{50}).

Fig. 7. Comparison of the 11,12-EET turnover kinetics of EH3, sEH and mEH. The substrate concentration-dependent reaction velocity for equal amounts of each enzyme is displayed. The calculation is based on the experimental data given in Tab. 1. While V_{max} dictates the reaction velocity at high substrate concentrations, in the concentration range far below substrate saturation (see inset) the catalytic efficacy is the important predictor. In consequence, the ratio of product formed during hydrolysis versus the substrate concentration is quite similar for all three enzymes at very low substrate concentrations and remains essentially constant with EH3 over the broad range displayed in the graph while it rapidly decreases with increasing substrate concentration in the case of mEH. The sEH displays an intermediate behaviour. The potential implications of these differences are discussed in the text.

Table 1. Catalytic properties of EH3 as compared to sEH and mEH

	EH3	sEH	mEH
8,9-EET			
V_{\max} [$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$]	12 ± 1	0.9 ± 0.1	0.12 ± 0.1
K_m [μM]	30 ± 3	1.7 ± 0.5	0.8 ± 0.25
k_{cat}/K_m [$\text{s}^{-1} \times \text{M}^{-1}$]	0.25×10^6	0.5×10^6	0.12×10^6
11,12-EET			
V_{\max} [$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$]	50 ± 5	4.5 ± 0.1	0.6 ± 0.03
K_m [μM]	80 ± 10	3.4 ± 0.3	0.4 ± 0.08
k_{cat}/K_m [$\text{s}^{-1} \times \text{M}^{-1}$]	0.4×10^6	1.4×10^6	1.2×10^6
14,15-EET			
V_{\max} [$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$]	60 ± 5	7 ± 0.3	0.04 ± 0.002
K_m [μM]	130 ± 10	15 ± 4	0.9 ± 0.1
k_{cat}/K_m [$\text{s}^{-1} \times \text{M}^{-1}$]	0.3×10^6	0.5×10^6	0.03×10^6
leukotoxin			
V_{\max} [$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$]	22 ± 0.5	0.55 ± 0.12	0.008 ± 0.0002
K_m [μM]	25 ± 0.6	1.5 ± 0.5	5.8 ± 0.2
k_{cat}/K_m [$\text{s}^{-1} \times \text{M}^{-1}$]	0.6×10^6	0.4×10^6	0.001×10^6

Data for V_{\max} and K_m are given as mean \pm SD and represent the average of 3 to 5 independent determinations. k_{cat}/K_m is calculated from the means of V_{\max} and K_m .

Table 2. Inhibition of EH3 catalysis by prototypic EH inhibitors

	EH3	sEH
Inhibitor [1 μ M]	Residual activity [% of solvent control \pm SD]	
1-(1-acetylpiperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea (TPAU)	7.2 \pm 1.4**	2.2 \pm 0.7**
12-(3-((3S,5S,7S)-adamantan-1-yl)ureido)dodecanoic acid (AUDA)	5.5 \pm 0.1**	1.6 \pm 0.5**
1-cyclohexyl-3-dodecylurea (CDU)	7.5 \pm 1.2**	2.0 \pm 0.3**
1-((3S,5S,7S)-adamantan-1-yl)-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea (AEPU)	18 \pm 0.7**	4.0 \pm 1.5**
8-(3-((3S,5S,7S)-adamantan-1-yl)ureido)octanoic acid (AUOA)	38 \pm 3.9**	14 \pm 2.6**
4-(((1R,4R)-4-(3-(4-(trifluoromethoxy)phenyl)ureido)cyclohexyl)oxy)benzoic acid (t-TAUCB)	97 \pm 3.3	0.8 \pm 0.1**
4-(((1S,4S)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid (c-AUCB)	97 \pm 4.6	0.2 \pm 0.1**
4-(((1R,4R)-4-(3-((3S,5S,7S)-adamantan-1-yl)ureido)cyclohexyl)oxy)benzoic acid (t-AUCB)	99 \pm 18	1.2 \pm 0.2**
1-((3S,5S,7S)-adamantan-1-yl)-3-cyclohexylurea (ACU)	103 \pm 10	8.1 \pm 2.2**
(E)-octadec-9-enamide (Elaidamide)	75 \pm 6.0	130 \pm 3.4*

Data are presented as mean \pm SD (n=3), compared to vehicle control.

* = $p < 0.05$; ** = $p < 0.01$













